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Fecapentaene concentration and mutagenicity in 718 North American stool samples

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Summary

The fecapentaenes (FP) are the predominant fecal mutagens identified to date, but they have not been shown to be carcinogenic. Epidemiologists looking for other fecal mutagens that may be related to colorectal cancer must disentangle from their investigations the pervasive mutagenic effect of the fecapentaenes. As a first step to studying the epidemiology of fecal mutagenicity independent of fecapentaenes, we compared FP measurements and Salmonella mutagenicity assay results for 718 acetone-extracted stool samples collected from a variety of subjects in the Washington DC metropolitan areas. In this large group, 50% of mutagenic samples contained elevated fecapentaenes. Specifically, three-quarters of the samples mutagenic in TA100 contained high FP levels. In contrast, mutagenicity in TA98 was not generally explainable by fecapentaenes, suggesting that non-fecapentaene TA98 mutagenicity should be one focus of future efforts to uncover colorectal carcinogens of etiologic importance.

Several investigators have reported that fecal mutagenicity is elevated in populations known to be at high risk for colorectal cancer (Ehrich et al., 1979; Mower et al., 1982; Reddy et al., 1985). Since mutagenicity is commonly used to screen for carcinogenicity, these observations have raised the possibility of identifying fecal carcinogens that cause colorectal cancer while in passage through the bowel. The best-characterized fecal mutagens are the fecapentaenes (FP), a family of ether-linked lipids produced by colonic bacteria from unidentified precursor compounds (Hirai, 1982; Van Tassell and Wilkins, 1986). Laboratory studies have demonstrated the substantial genotoxicity of the fecapentaenes (Plummer et al., 1986; Curren et al., 1987; Schmid et al., 1987), but two lines of

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evidence argue against the simple hypothesis that elevated fecapentaene excretion increases the risk of colorectal cancer. First, carcinogenicity experiments in rodents have yielded no excess of tumors (Ward et al., 1988). Second, patients with colorectal cancer excrete lower amounts of fecapentaenes than matched controls (Schiffman, 1987).

Since the fecapentaenes do not appear to be carcinogens, we are now focusing on non-fecapentaene fecal mutagenicity in our epidemiologic studies of colorectal cancer. Specifically, we are looking for other mutagens that may be elevated in colorectal cancer cases. This search is complicated by the potency and seemingly wide prevalence of the fecapentaenes (Dion and Bruce, 1982), whose mutagenic effect must be controlled for in our current investigations. As a first step in studying the epidemiology of fecal mutagenicity independent of fecapentaenes, we compared fecapentaene measurements and Salmonella mutagenicity assay results for 718 stool samples collected in our investigations in the Washington DC metropolitan area. Through this comparison, we sought to determine what percentage of the mutagenicity detected by our extract/assay methods could be explained by high fecapentaene concentration. As a corollary, we wished to know what percentage of samples appeared to contain unidentified, non-fecapentaene mutagens that might merit further study.

Methods

The 2-day stool samples collected for this investigation came from 296 subjects, who each collected from one to four 2-day pooled samples onto dry ice at home. 24 samples (3%) were excluded due to inadequate material, leaving 718 for analysis. The subjects were participants in our investigations of colorectal cancer and fecal mutagenicity, and included the following groups: 76 subjects had newly-diagnosed adenocarcinomas or large adenomas of the colon or rectum, 114 were undergoing elective surgery for non-gastrointestinal, non-oncologic conditions, and 106 were undergoing colorectal diagnostic workup and were found not to have clinically significant neoplasia. For this analysis comparing fecapentaene levels to mutagenicity results, the 3 study groups were merged. Overall, 60% of the 296 study subjects were male, 86% were white, and the median age of the group was 61 years (range 25–89).

The samples were obtained as follows: For each 2-day collection, the subjects were given a styrofoam chest containing dry ice. They collected stool into a plastic container held by a collection bonnet placed on the toilet rim. The container was placed immediately into the dry ice chest. Subjects were shown how to use the collection kits to avoid urine contamination, and this proved not to be a problem. The number of stools making up a pooled sample ranged from one to seven (average 2–3). Stools were freeze-dried in their individual containers without thawing. The lyophilates were pooled and mixed for the entire two-day collections, then stored at $-40\,^{\circ}C$ or colder in sealed, air-tight containers. Aliquots of the samples were sent in screw-top vials in batches on dry ice to the two testing labs. As a quality control measure at the end of the study, 1 out of every 20 samples was resubmitted in a masked fashion for repeat assay by both labs. Mutagenic samples were purposely over-represented among the repeats.

Fecapentaenes were measured at the Anaerobic Microbiology Laboratory of VPI (Blacksburg, VA). 1-g samples of the freeze-dried materials were extracted and analyzed by high-performance liquid chromatography (HPLC), as previously described (Schiffman et al., 1988). Briefly, freeze-dried samples were extracted in 20 ml acetone supplemented with butylated hydroxytoluene (BHT). Each extract was vacuum-filtered, evaporated under vacuum at $60\,^{\circ}C$, stoppered under argon, and placed in ice. The evaporated extracts were (i) resuspended in 0.5 ml of prechilled HPLC solvent, (ii) filtered through an Acrodisc LC-13 filter (0.5 μm) into 1-ml septum-capped vials and (iii) sealed under argon.

We determined the concentration of total fecapentaenes in each extract on a Waters liquid chromatograph using a Radial Compression Module and 8 mm (5 micron) silica cartridges, with chloroform/isopropanol (95 : 5) containing 50 μg/ml BHT. The flow rate was 2 ml/min. The HPLC was calibrated using dilutions of known concentrations of synthetic fecapentaene-12; the

peak areas of these standards were then correlated with those of the experimental samples. Absorbance was monitored at 365 nm on a Waters 440 UV Detector (AUFS 0.05) and peak areas were integrated on a Hewlett Packard 3390A recording integrator. The reading corresponded to "total fecapentaenes", without separation of fecapentaene-12 and fecapentaene-14 (Baptista et al., 1984).

For the statistical analysis shown, fecapentaene concentration was categorized into three groups: "low" = less than 100 ng/g stool (non-integrable or no peak on HPLC), "intermediate" = 100–999 ng/g, and "high" = $\geq$ 1000 ng/g. A sample containing 1000 ng FP/g would yield 0.25 μg FP per screening plate in the mutagenicity assays, a dose in the range known to increase the number of induced revertants in testing of synthetic racemic fecapentaene-12 (Goggelmann et al., 1986).

Salmonella/mammalian microsome mutagenicity assays were conducted at the Microbial Mutagenesis Screening Laboratory of the NCI Frederick Cancer Research Facility (Frederick, MD). Samples were stored under nitrogen at $-20\,^{\circ}$C until needed. To extract each sample, 2.5 g was added to 50 ml of acetone and the suspension was shaken for 30 min at room temperature. The suspensions were vacuum-filtered and the filtrate evaporated using a rotary evaporator with the water bath set at $50\,^{\circ}$C. For the initial screening tests, 1.5 ml of acetone was added and the flask hand-shaken. The suspended residue was added in 0.15 ml aliquots so that each plate contained 250 mg equivalent of the original lyophilized stool sample.

The plate-incorporation assays were performed as recommended by Ames et al. (1975) with the modifications of Andrews et al. (1978). The stock cultures of TA98 and TA100 were maintained as frozen permanents at $-80\,^{\circ}$C, and fresh 15-h cultures were grown in Oxoid No. 2 nutrient broth on a reciprocating shaker at $37\,^{\circ}$C. For the preparation of S9 mix, male syrian golden hamsters were induced with 500 mg/kg of Aroclor 1254 (one dose suspended in corn oil injected i.p. 5 days before sacrifice by decapitation). Each plate with S9 mixture contained 3 mg of protein. Each strain was tested with and without the addition of the S9 mix. All experiments were conducted in duplicate under yellow lighting, with appropriate negative controls (cells only and acetone plus cells) and positive controls (2-nitrofluorene). Duplicate plates were incubated at $37\,^{\circ}$C for 48 h and revertant colonies were counted using a hand held tally. The mean number of revertants for duplicate plates was compared to the mean number of revertants for the negative controls.

When a test sample yielded at least $1.5\times$ the mean number of spontaneous revertants (the background), a dose response was constructed using four doses of 50, 100, 200 and 400 mg equivalents of lyophilate. A sample was judged mutagenic if two consecutive doses yielded at least twice the background. Samples screening positive (at $1.5\times$ background) but not judged definitely mutagenic were termed "borderline". The results were compiled for each of the four assay combinations (TA98, TA98 + S9, TA100, TA100 + S9) and, in addition, the strongest response obtained with any assay was tabulated. For statistical analyses, borderline results were grouped as non-mutagenic.

A chi-square analysis was performed to test the statistical significance of associations seen between mutagenicity results (non-mutagenic and borderline combined versus mutagenic) and fecapentaene levels (low, intermediate, high). Because many individuals contributed more than one sample, the chi-square statistics could not be assumed to have the chi-square distribution under the null hypothesis of no association. We therefore used a bootstrap procedure (Efron and Tibshirani, 1986) to develop a test that accounted for the dependence of samples from the same individuals. We randomly sampled with replacement from the 296 individuals such that each of 200 replicate bootstrap data sets had the same number of individuals contributing 1, 2, 3 or 4 samples as the original data set. We then performed the regression version of the 2 degrees of freedom test of association for each replicate data set, and used the empirical variance of the regression slopes in a Wald test (Rao, 1973) to generate correctly conservative p-values for the chi-square statistics shown in the results. Finally, to confirm the results, we repeated the analysis deleting all but the first sample obtained from each individual and separately examined subgroups of the study population defined by age, race, sex and clinical presentation.

Results

The fecapentaene and mutagenicity assays were reliable, as indicated by the 36 quality control repeats. The Spearman rank correlation coefficient comparing first and replicate fecapentaene measurements was 0.91. Early measurements completed up to two years before the quality control batch were replicated as closely as recent values, indicating that there was no significant laboratory drift or degradation of samples over time. The percentage of exact agreement for the repeated mutagenicity assays was 78% for TA98, 89% for TA98 + S9, 89% for TA100, 86% for TA100 + S9, and 80% for the strongest response obtained by any assay. All but one disagreement between original and replicate mutagenicity results represented single-category misses, e.g., a sample called borderline on original assay called non-mutagenic or mutagenic when repeated. A single sample tested non-mutagenic on TA98 originally but was mutagenic on repeat.

The comparisons of mutagenicity results and fecapentaene levels for all 718 samples are given in Table 1. Overall, of the 56 samples mutagenic in at least one assay, one-half contained elevated levels of fecapentaenes, a highly significant association ($p < 0.01$). Specifically, we found that TA100 mutagenicity was very strongly associated with fecapentaene concentration; 74% of the samples mutagenic in TA100 without S9 contained at least 1000 ng FP/g and 80% of the samples positive in TA100 plus S9 contained at least 1000 ng FP/g ($p < 0.01$ for both assay combinations). In contrast, TA98 mutagenicity was not significantly related to high levels of fecapentaenes: only 39% of samples mutagenic in TA98 and 12% of samples mutagenic in TA98 plus S9 contained over 1000 ng FP/g.

Although borderline results were grouped as non-mutagenic in the main analysis, samples testing borderline using TA98, TA100 and TA100 plus S9 appeared to be associated with elevated fecapentaenes. When borderline results were grouped as mutagenic, the association of TA98 mutagenicity with FP levels became statistically significant ($p < 0.01$). Regardless of the grouping of borderline results, TA98 plus S9 appeared unrelated to fecapentaenes.

Non-fecapentaene mutagenicity was uncom-

TABLE 1

FECAPENTAENE CONCENTRATION AND MUTAGENICITY IN 718 STOOL SAMPLES

Fecapentaene concentration (ng/g dry stool)	Mutagenicity result, by assay														
	TA98			TA98+S9			TA100			TA100+S9			Any [a]		
	− [b]	+/−	+	−	+/−	+	−	+/−	+	−	+/−	+	−	+/−	+
<100	257 (40%)	8 (18%)	12 (39%)	260 (38%)	14 (50%)	3 (38%)	274 (41%)	2 (09%)	1 (03%)	275 (39%)	2 (18%)	0 (00%)	244 (41%)	18 (30%)	15 (27%)
100–999	222 (35%)	14 (32%)	7 (23%)	233 (34%)	6 (21%)	4 (50%)	230 (35%)	5 (23%)	8 (23%)	240 (34%)	1 (09%)	2 (20%)	212 (35%)	18 (30%)	13 (23%)
1000+	164 (26%)	22 (50%)	12 (39%)	189 (28%)	8 (29%)	1 (12%)	157 (24%)	15 (68%)	26 (74%)	182 (26%)	8 (73%)	8 (80%)	145 (24%)	25 (41%)	28 (50%)
Total	643 (100%)	44 (100%)	31 (100%)	682 (100%)	28 (100%)	8 (100%)	661 (100%)	22 (100%)	35 (100%)	697 (100%)	11 (100%)	10 (100%)	601 (100%)	61 (100%)	56 (100%)
	$\chi^2 = 2.7$, $p = 0.50$ [c]			$\chi^2 = 1.3$, $p = 0.75$			$\chi^2 = 42.6$, $p < 0.01$			$\chi^2 = 14.6$, $p < 0.01$			$\chi^2 = 15.3$, $p < 0.01$		

[a] Strongest response obtained in any of the 4 assays.

[b] "−" mean non-mutagenic, "+/−" means borderline, "+" means mutagenic.

[c] χ^2 values computed with borderline and non-mutagenic categories combined; p values corrected for replicate measurements by bootstrap simulation described in text.

mon in the study population, using the extract/assay methods we chose. Among the 520 samples containing less than 1000 ng FP/g, only 5% were mutagenic in any assay.

Although mutagenicity in the samples seemed to derived commonly from fecapentaenes, the mutagenicity assays were not an adequate screen for high excretors. Of the 198 samples with fecapentaenes over 1000 ng/g, only 53 (27%) screened positive or even borderline as the strongest response obtained by any assay. Among the 48 samples containing over 4000 ng FP/g (equivalent to 1 μg/plate), 24 (50%) still screened negative.

When we restricted the analysis of the first sample obtained from each individual, the results were unchanged although the statistical significance of some findings was reduced by the smaller numbers. However, when we examined separately the three clinical groups pooled to create the study population, mutagenicity was not associated with fecapentaene levels in the patients with colorectal neoplasia. These patients demonstrated very little TA100 mutagenicity, in keeping with their lowered fecapentaene excretion (Schiffman, 1987). Unlike the other two groups, the fecal mutagenicity observed in the patients with colorectal neoplasia was detected mainly in TA98 without S9, an intriguing finding that we are pursuing in subsequent work.

As a final topic, we subdivided the samples according to the age, sex and race of the donors and repeated the analyses within these subgroups. The strong association of mutagenicity and fecapentaene concentration was seen in all ages and in both sexes, but was apparent only for whites. Non-whites had about the same percentage of samples mutagenic with any assay (7%) as whites (8%), but none of the mutagenic samples from non-whites contained greater than 1000 ng FP/g and only one was positive with TA100.

Discussion

The results of this epidemiologic investigation extend the findings of Dion and Bruce (1983), who observed in a small group of 24 donors that most fecal mutagenicity extracted by acetone could be attributed to a lipid-soluble fraction later shown to contain the fecapentaenes. Our results from a much larger population suggest that virtually all of the fecal mutagenicity detected in acetone extracts by Salmonella tester strain TA100 may relate to increased fecapentaenes, with few other mutagens apparent in the low-fecapentaene samples. On the other hand, Salmonella strain TA98 detected mainly non-fecapentaene mutagens (especially when S9 mix is added); these mutagens were seen in 5% or less of acetone-extracted samples from our population.

The results indicate that when fecapentaenes are the object of study, HPLC measurements must be used, since the mutagenicity assays failed to detect even the majority of samples with high fecapentaene concentrations. Inhibitors of mutagenicity have been documented in fecal samples and may be responsible for this observation (Bruce et al., 1977; Hayatsu et al., 1981). If fecapentaene mutagenicity is of interest, Salmonella TA100 alone may be sufficient; TA98 detected only one sample with high fecapentaene concentration missed by TA100.

When fecal mutagens other than the fecapentaenes are the object of study, the investigator must choose an approach to avoid or account for their pervasive effect. If acetone or other mid-polarity solvents known to extract fecapentaenes are used, the assay could be limited to TA98 plus S9 or another system found to be insensitive to the fecapentaenes. The number of mutagenic samples would be expected to be reduced. Alternatively, other solvents could be chosen that do not as effectively extract fecapentaenes, such as water or hexane : ether. This approach has been taken by at least three groups (Kuhnlein, Kuhnlein and Bell, 1983; Shaw et al., 1985; Reddy et al., 1985) and we eventually plan to test our stored samples using such solvents. For each, it will be necessary to document the relative absence of fecapentaenes in the extracts since these data are not generally available. One problem with this approach may be that no single mutagen has yet been identified in any solvent to rival the fecapentaenes in prevalence and concentration. Less prevalent mutagens will be more difficult to study in epidemiologic studies, requiring larger numbers of subjects to provide an adequate number of positives. Less concentrated mutagens, such as the potent aromatic amines derived from cooking meat, may

be detected best using special extraction or assay methods (Felton et al., 1986; Hayatsu et al., 1986).

As a final approach to separating fecapentaene and non-fecapentaene mutagenicity in population-based studies, fecapentaenes can be measured directly by HPLC for each sample concurrently with the mutagenicity testing, as was done here. Mutagenic samples containing low fecapentaenes thus can be studied as a discrete category. We are taking this approach in current investigations, in which we are studying dietary and other influences on fecal mutagenicity, as well as the possible association of non-fecapentaene TA98 mutagenicity with risk of colorectal cancer.

A few interesting related points are raised by our data. It proved useless (and costly) to include tester strain TA100 plus S9 in the investigation, since no additional mutagenicity missed by TA100 alone was detected by this combination. In contrast, 16 additional mutagenic samples were detected by the addition of TA98 and yet another 5 samples were mutagenic only in TA98 plus S9.

The apparent association of high fecapentaene concentrations with some "borderline" mutagenic samples demonstrates that even a 1.5-fold elevation in revertants can be meaningful. We plan to continue to use low screening thresholds in future studies followed by dose–response evaluations.

Finally, it will be interesting to see if non-white North Americans truly excrete a different spectrum of mutagens than whites, as suggested by our data. Such a finding, if confirmed, might shed some light on the origins and determinants of fecal mutagenicity, which are still poorly understood.

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